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Estetrol: A unique steroid in human pregnancy

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ABSTRACT

Estetrol (E_4) is an estrogenic steroid molecule synthesized exclusively by the fetal liver during human pregnancy and reaching the maternal circulation through the placenta. Its function is presently unknown. After its discovery in the mid-1960s, E_4 research revealed rather unique properties of this steroid and spawned a large body of state-of-the art publications. Nevertheless, 20 years later experimental work was virtually abandoned.

In recent years based on new data, E_4 has experienced a *vita nova*, a revival of preclinical and clinical research activities with the goal to elucidate its physiological function and explore its potential for therapeutic use in humans.

This review is intended to offer an historical account of the discovery of E_4 and the preclinical studies conducted during the heyday of E_4 research that ended in the mid-1980s.

1. The discovery of estetrol

After a period of heightened research activities, followed by an extended hiatus, experimental interest in E_4 has now been rekindled [1,2]. The first suggestion of a novel estrogen metabolite, later identified as estetrol (E_4), emerged in the mid-1960s as part of an experiment to investigate the metabolism of estradiol (E_2) in early infancy. In that experiment, Diczfalusy and co-workers [3] administered ^{14}C-E_2 intramuscularly to three 6 to 13-week old infants born with multiple malformations and measured estrogenic metabolites in four 24-h urine specimens collected from each of the subjects. They reported that estrone (E_1) and E_2 accounted for about 4% and estriol (E_3) for approximately 12% of the total urinary radioactive material. The surprising finding was the presence of a novel compound, which the authors described as follows:

> Perhaps the most striking finding of the present study is the detection of a new and major E_2 metabolite, which on the average accounted for 16% of the total urinary radioactive material. Although it was not identified completely, evidence was obtained indicating that it is a phenolic steroid possessing probably four acylable hydroxyl groups but no oxo-groups. Since this metabolite formed.

An acetonide, it must contain 2 adjacent hydroxyl groups with the same steric orientation. It is unlikely that the compound contains an additional hydroxyl group in ring A. As this compound is phenolic, if the assumption is made that it is a tetrol with a 17β-hydroxyl group, then the other two hydroxyl groups must be in positions 16α and 15α, or in positions 16α and 18.

In this remarkable piece of early scientific detective work, the authors correctly hypothesized on the presence of two adjacent hydroxyl groups, proposed the same steric configuration for these two groups, and suggested the 16α and 15α positions as one possibility.

Soon thereafter, Gurpide et al. [4] examined the fetal and maternal metabolism of E_2 during pregnancy by isotope dilution methods, simultaneously administering tritiated (^3H-) and ^{14}C-E_2. Tritiated E_2 was administered intra-amniotically or intraperitoneally and ^{14}C-E_2 was injected at the same time into a peripheral maternal vein. The authors found a urinary metabolite more polar than E_2. By comparing infrared spectra, they concluded that their compound was identical to that detected by Diczfalusy and co-workers. They named the compound estetrol.

During the same period, Diczfalusy and co-workers [5] succeeded in isolating and identifying the novel estrogen by extracting 200 L of late pregnancy urine. On the basis of physical and chemical characteristics they concluded that the compound was identical with 15α-hydroxyestriol (15α-OHE_3) or estra-1,3,5(10)-triene-3,15α-,16α-,17β-tetrol. They further concluded that its origin was the fetal liver, based on their previous work, which showed that the

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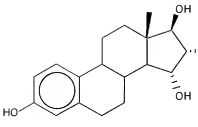

Fig. 1. Structural formula of estetrol (Estra-1, 3, 5(10)-triene-3, 15α, 16α, 17β-tetrol).

liver is the exclusive site of 15α- and 16α-hydroxylation [6,7,8]. The structural formula of estetrol is presented in Fig. 1.

2. Human studies

2.1. Site of estetrol production

Estetrol is a pregnancy hormone produced by the fetal liver. It was found in maternal urine as early as week nine of pregnancy [9,10]. The steroid was shown to be of fetal origin by isotope dilution methods involving the injection of ^{3}H-E_2 into fetal compartments (intra-peritoneally or intra-amniotically) and ^{14}C-E_2 into a maternal vein. Analysis of estrogenic metabolites in maternal urine showed minimal ^{14}C-E_4. Estetrol was found predominantly as the tritiated compound (the isotope infused directly into the fetus), thus revealing the fetal origin of maternal E_4 [4].

The site of E_4 synthesis was identified to be the fetal liver because only the fetal liver is capable of both 15α- and 16α-hydroxylation, as shown by Schwers et al. [7] who perfused two previable fetuses (20th week of gestation) with a combination of ^{3}H-E_1 and ^{14}C-E_1 sulfate. Analysis of the radiolabeled metabolites showed that 15α- and 16α-hydroxylation took place mainly, if not entirely, in the fetal liver, and in none of the other tissues examined (adrenal, intestine, lung, and pooled residual fetal tissues). These findings are consistent with another study by the same group [11] showing that E_1 and E_2 are converted to E_3 exclusively by the fetal liver. Both studies found that the conversion of the steroids was preceded by their conjugation. Another report by this group [6] provided further evidence for the 15α-hydroxylation capacity by the fetus. Following injection of ^{3}H-E_1 and ^{14}C-E_2 into the intact feto-placental unit in situ, a novel compound was detected and characterized as conjugated 15α-OHE_2.

In a later study by this group [8], previable fetuses (week17–21) were perfused with ^{3}H-testosterone (T) plus ^{14}C-androstenedione (AD) or with ^{3}H-dehydroepiandrosterone (DHEA) plus ^{14}C-AD. In addition, liver, adrenal, and other tissues (the latter combined into two pools) were obtained from fetuses that had been perfused with ^{3}H-AD. These tissues were incubated with ^{14}C-AD. Among all tissues, only the fetal liver was capable of producing 15α- and 16α-hydroxylated AD and T, preceded by aromatization. Cantineau et al. [12] incubated several steroidal precursors with fetal liver microsomes and isolated both 15α- and 16α-hydroxylated products, including E_4, thus directly demonstrating 15α- and 16α-hydroxylating enzymatic activities in liver microsomes.

2.2. Metabolic precursors of estetrol

Various phenolic and neutral steroidal precursors have been administered to human volunteers in an effort to elucidate the metabolic pathways in the synthesis of E_4. The steroids were labeled either with ^{3}H or ^{14}C. These studies typically involved the injection of either one or two radiolabeled compounds of established stability and the presence of each isotope can be measured simultaneously in plasma or urine.

Two studies examined the metabolic fate of simultaneously injected ^{14}C-E_2 and ^{3}H-E_3 to evaluate the contribution of each to the formation of E_4. In one study [13] the hormones were administered intra-amniotically to a subject who at week 14 of gestation underwent therapeutic abortion. In the other study [14] ^{14}C-E_2 and ^{3}H-E_3 were administered to an anencephalic fetus via the umbilical vein 40 h after birth. Urinary metabolites were measured up to 72 h after injection. Estetrol was found to be the major metabolite of both E_2 and E_3. Because the most abundant urinary product after radiolabeled E_2 administration was E_4 [13], it was concluded that E_2 was the major precursor of E_4—although with time E_3 was also metabolized to E_4 [14]. The results from a third study are consistent with these findings. In that study, ^{14}C-E_2 and ^{3}H-15α-hydroxyandrostenedione (15α-OHAD) were simultaneously transfused into a fetus in utero for erythroblastosis fetalis and E_4 was derived at approximately equal amounts from E_2 and 15α-OHAD [15].

Of particular interest was the question whether 15α-OHE_2 is metabolized to E_4 by 16α-hydroxylation. To this end, ^{3}H-15α-OHE_2 was infused into two fetuses and 11% and 4% of the total injected tritium was found to be E_4 in maternal urine [15]. When 15α-OHE_2 was given intravenously to healthy volunteers during the third trimester of pregnancy in three studies [15,16,17], E_4, excreted as Ring D monoglucuronide, was found in the urine of all women, supporting the conclusion that 15α-OHE_2 can serve as a precursor of E_4.

Two studies examined the role of neutral steroids as precursors of E_4 when administered intravenously as radiolabeled compounds to healthy volunteers during the third trimester of pregnancy [18,19]. The administered steroids were 16α-hydroxydehydroepiandrosterone (16α-OHDHEA); 16α-OHDHEA sulfate; DHEA sulfate; 15α-OHDHEA, 16α-hydroxyandrostenedione (16α-OHAD) and 15α-OHAD. Following injection of these compounds into maternal peripheral veins, E_4 was isolated in maternal urine at low concentrations in all cases. Another study of neutral steroids examined the metabolic fate of 15α-OHAD and DHEA sulfate when directly delivered to the fetal circulation during a transfusion in utero for erythroblastosis fetalis [20]. Analysis of maternal urine revealed that both steroids were converted to E_4. However, 15α-OHAD was converted to E_4 at considerably better yields than was DHEA sulfate. A schematic outline of the pathways of E_4 is presented in Fig. 2.

The interpretation of the physiologic significance of injected precursors in the synthesis of E_4 is complicated by a number of factors. For example, the levels of urinary metabolites following injection of the precursors are influenced by the endogenous concentration of the corresponding precursors. Further, the formation of E_4 from precursors injected into the maternal circulation depends on the extent to which the precursors reach the fetal circulation for conversion to E_4 in the fetal liver. Nevertheless, the available data support the following conclusions: (1) E_4 can be formed both from phenolic and neutral precursors; (2) the phenolic pathway appears to be the more important pathway [12]; (3) E_2 is a major precursor of E_4 [13,14,15]; (4) E_4 precursors are first sulfoconjugated prior to 15α- and 16α-hydroxylation [7,11,12]; and (5) E_4 is formed via first 15α- and then 16α-hydroxylation; the reverse sequence is of minor importance [18].

2.3. Excretion of estetrol

After administration to adults, E_4 is conjugated but otherwise not further metabolized. It is excreted rapidly and completely in urine. After administration of ^{3}H-E_4 to two postmenopausal women, Fishman found 85.0% and 73.0% of the injected radioactivity excreted in urine within the first 24 h. Virtually the entire urinary radioactivity represented unmetabolized E_4 [21]. Although

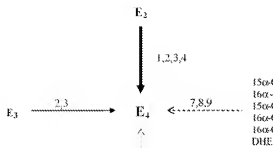

Fig. 2. Schematic outline of estetrol synthesis based on urinary excretion of metabolic precursors. The relative importance of E4 precursors is indicated by the thickness of the arrows. Whether and to what extent each of the listed steroids represent physiologically significant E4 precursors remains unclear because the compounds were exogenously administered and their metabolic conversion may therefore not be entirely representative of endogenous metabolic pathways. *Abbreviations*: E2 = estradiol; E3 = estriol; E4 = estetrol; 15α-OHE2 = 15α-hydroxyestradiol; 15α-OHAD = 15α-hydroxyandrostenedione; 15α-OHDHEA = 15α-hydroxydehydroepiandrosterone; 16α-OHDHEAS = 16α-hydroxydehydro- epiandrosterone sulfate; DHEAS = dehydroepiandrosterone sulfate. *References* (1) Gurpide et al. [4]; (2) Schwers et al. [13]; (3) Hagen [14]; (4) Schut et al. [15]; (5) Jirku et al. [16]; (6) Nagatomi et al. [17]; (7) YoungLai and Solomon [18]; (8) Stanczyk and Solomon [19]; (9) YoungLai et al. [20].

not calculated in that study, the data are consistent with a metabolic half-life of 12–15 h. Further analysis of the excretion products from that study indicated that E_4 was predominantly glucuronidated on Ring D [22].

A study in third-trimester pregnant women [23] found qualitatively similar urinary excretion patterns to those of nonpregnant women. After administration of 3H-E_4, urinary E_4 was identified as glucuronidate but otherwise unmetabolized. However, only 64–77% of the injected radioactivity was recovered in urine during the 4-day study period, in contrast to the two postmenopausal women, in whom 95% and 98% was recovered during the same period [21].

2.4. Maternal and fetal estetrol levels during pregnancy

As specific radioimmunoassays for E_4 were developed [24–29] it became possible to quantitate plasma levels, and a number of laboratories measured the levels of E_4 in maternal and fetal plasma and amniotic fluid. Table 1 summarizes reported results of unconjugated serum E_4 concentrations in the maternal and fetal circulation. Fig. 3 illustrates the rising E_4 levels during pregnancy.

As a whole, the results of these studies indicate that, on average, the late-pregnancy maternal E_4 concentrations are approximately in the 3 nanomolar levels (~1 ng/mL). The differences in mean E_4 values observed in different studies at equivalent periods of pregnancy may in part be attributed to different specificities of antisera used in the radioimmunoassays [30].

One study [28] measured conjugated and unconjugated maternal plasma E_4 and found conjugated (glucuronated) E_4 at concentrations about seven times higher than unconjugated E_4 in third-trimester maternal plasma (4.57 ng/mL vs. 0.67 ng/mL during week 37–40). If this ratio is representative, then the total E_4 levels may be considerably higher than the unconjugated levels that are generally reported (summarized in Table 1).

Starting around week 30, the E_4 plasma levels increase substantially as pregnancy progresses [31,32]. One study reported 7-fold higher values at week 40 when compared to week 22–26 [33]. The increases in E_4 occur more rapidly than those in E_3 both in plasma [33,34] and in total amounts excreted in urine over 24 h [10]. Estetrol levels in the fetus are substantially higher than those in the mother. One study reported fetal levels at term to be 12 times higher than maternal levels [33], another study found fetal levels nearly 19 times higher than those in maternal plasma, i.e., 18.63 ng/mL vs. 1.00 ng/mL [35].

Unconjugated E_4 was also found in amniotic fluid at levels of 7.3 ng/mL [33], in agreement with an earlier study [36] that reported between 5 and 6 ng/mL (estimated from Fig. 8 of the report). A study in twin pregnancies found levels of conjugated E_4 in amniotic fluid that were approximately six times higher than those

Table 1
Mean levels of unconjugated estetrol (ng/mL) in maternal plasma at different stages of pregnancy

Source	Mean value (ng/mL)	Time of measurement
Fishman and Gurpide [25]	1.84	3rd trimester
Giebenhain et al. [34]	0.45[a]; 2.20	Week 24; 40
Tulchinsky et al. [33]	0.17; 1.20	Week 22–26; 40
Korda et al. [31]	~2.0; ~5.0	Week 20; term
Tulchinsky et al. [48]	0.8[a]	Week 35–40
Kundu and Grant [26]	0.23[a]; 0.83[a]	Week 29–30; 39–40
Den et al. [28]	0.13; 0.67	Week 25–28; 37–40
Notation and Tagatz [40]	0.63; 2.20	Week 31, 40
Belisle et al. [49]	0.95[c]	Week 36–38
Kundu et al. [30]	0.14[a]; 0.90[a]	Week 29–30; 39–40
Axelsson [50]	0.19; 0.61	Week 33–34; 39–40
De Cecco et al. [51]	0.10[b]	3rd trimester
Guadandi et al. [52]	0.51[b]	Week 39
Künzig et al. [53]	0.25; 1.28	Week 22; end of pregnancy
Pedretti et al. [54]	0.34[a]	3rd trimester
Künzig et al. [32]	0.27; 1.37	Week 22; term
Kundu et al. [35]	0.94; 1.00	Week 38–41; labor
Kundu et al. [55]	1.08[a]; 0.99[c]	Antepartum; labor
Lupo et al. [56]	0.62	3rd trimester
Schollberg et al. [57]	0.41	Week 32–38

[a] Estimated from figure.
[b] Median value.
[c] Same subjects, antepartum blood was drawn 1–6 days before labor.

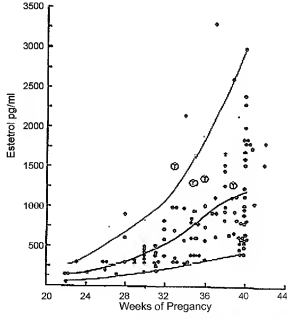

Fig. 3. Plasma estetrol throughout pregnancy (Reproduced with permission from Tulchinsky et al. [33]).

of unconjugated E_4. The levels of unconjugated and conjugated E_4 did not differ significantly between twins, and did not differ before or during labor [37].

Total maternal urinary excretion of E_4 increased from 0.28 mg/24 h at week 20 to 2.09 mg/day at week 40 of pregnancy [38]. Another study reported levels of 2.28 mg/day during weeks 36–40 [39]. In contrast, two studies found considerably lower third-trimester urinary excretion levels of E_4, i.e., about 0.52 [18] and 0.5 mg/day [23].

2.5. Maternal estetrol levels as an index of pregnancy complications

The discovery of E_4 generated great enthusiasm regarding its potential as an index to identify and survey complicated pregnancies. As a hormone of exclusively fetal origin, E_4 was considered especially suitable to survey the status if the intra-uterine fetus during pregnancies complicated by pathological conditions and/or fetal abnormalities. Accordingly, six studies [30,32,33,38,40], were designed to measure circulating E_4 levels in maternal plasma during various conditions of pregnancy abnormalities, including Rh isoimmune disease, diabetes mellitus, recurrent intrahepatic cholestasis of pregnancy, toxemia, and intrauterine fetal death. The results from these studies revealed that E_4 was of limited value in pregnancy monitoring because it did not represent a unique specific marker for the identification of fetal abnormalities. For follow-up and survey of pregnancy pathology E_4 levels were not suitable due to the large intra- and inter-individual variation of plasma levels.

3. Preclinical studies

3.1. Rodent uterus

A number of studies were conducted to examine physiologic effects of E_4 on the uterus. One study [41] exposed immature rats to several estrogens in paraffin pellets implanted subcutaneously. The pellets weighed 5–6 mg and contained 10% by weight of each of the estrogens under study. Uterine wet weights and dry weights were measured 24, 48 and 72 h after pellet implantation. Significant increases were observed in these parameters following treatment with E_1, E_2 and E_3. The increases in wet and dry weight in response to these hormones started at 24 h and continued up to 72 h. In contrast, E_4 treatment had no uterotrophic effects.

Another study [42] evaluated uterine weight and several additional parameters in response to E_4 treatment of immature rats. The following compounds, in μg/100 g body weight, were injected subcutaneously in arachis oil: $E_2 = 1$ μg, $E_3 = 1$ μg, $E_4 = 10$ μg or 50 μg. Significant increases in uterine wet weight and luminal fluid were observed in all treatment groups as early as 6 h after injection, but in contrast to E_2 and E_3, the short-term increases in wet weight were not sustained after E_4 treatment. Following E_2 (but not E_3 or E_4), uterine protein content and alkaline phosphatase activity increased significantly at 24 h and remained elevated at 36 h. To evaluate more sustained (48 h) effects of E_4, the same compounds were injected twice, at time 0 and 24 h. Following this treatment, E_4 produced significant increases in uterine weight, protein content and alkaline phosphatase activity, but not in DNA content. Thus, the study revealed rapid responses of the uterus to E_4. The short-term (6 h) E_4 effects on uterine weight gain were comparable to those of E_2 and E_3; they very likely can be attributed to water retention (imbibition) and were no longer observed at 24 h or thereafter. The absence of DNA increases suggests that E_4 preferentially stimulated functional parameters (alkaline phosphatase activity, protein synthesis) instead of growth (mitotic) parameters. In contrast, E_2 produced substantial increases in total uterine DNA to nearly three times the pretreatment levels.

The uterine effects of E_4 were further characterized by evaluating changes in progesterone receptors and histologic and ultrastructural features of the immature rat uterus in response to E_4 [43]. Tamoxifen (TAM) was included in these studies as an additional comparator. Immature rats were treated in 24 h intervals with three subcutaneous injections of the test compounds in arachis oil at the following doses: E_2 and $E_3 = 1$ μg; and E_4 and TAM = 50 μg, each per 100 g body weight. Tissues were examined 72–75 h after the first injection, i.e., 24–27 h after the third injection. Estetrol produced small but statistically significant increases in uterine weight to 55% above the pretreatment levels compared to 300% after E_2 treatment at a 50× lower dose of E_2. Increases in response to E_3 and TAM were somewhat higher than those produced by E_4, but substantially lower than those in the E_2 group. Estetrol significantly increased total protein/mg DNA, cytosol protein/mg DNA and progesterone receptor levels/mg DNA. In addition, E_4 produced distinct histologic effects when examined by light and

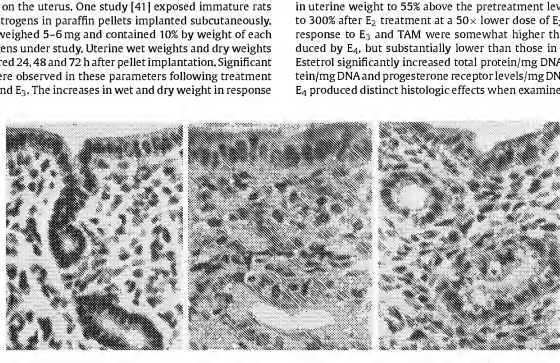

Fig. 4. Effects of estetrol on the immature rat uterus Sections (480×) show surface (visible on top), glandular and stromal cells. Estetrol (right panel) produced low columnar surface and glandular epithelial cells with a higher cytoplasmic/nuclear ratio than that of control tissue (left panel), which showed mostly cuboidal epithelium with nuclei making up most of the cell volume. The stroma of E4 tissues was relatively dense when compared to control and E2 tissues (center panel). The cytoplasmic/nuclear ratio of surface and glandular epithelial cells was highest after E2 treatment. Necrotic cells were seen both in E4 and E2 (arrow) tissues. (Reproduced with permission from Holinka et al. [43]).

electron microscopy. The changes in uterine histology induced by E_4, compared with control and E_2 treatment, are shown in Fig. 4.

The studies by Holinka et al. [42,43] document the estrogenicity of E_4 when the immature rat uterus is used as a model. In contrast, Martucci and Fishman [41] did not detect any uterotrophic activity of E_4. This may reflect lower levels of E_4 administered in their study, where the compound was dissolved in paraffin pellets that were implanted subcutaneously. In contrast, Holinka and co-workers administered E_4 in arachis oil, which spread over large subcutaneous areas and thus enhanced absorption. In addition, the amount of E_4 in the Martucci and Fishman study was equal (per weight) to that of E_2, whereas Holinka and co-workers used E_4 at 10× or 50× the E_2 doses.

3.2. Studies in cell culture

Jozan and Kreitmann [44] examined the effects of E_4 when compared to those of E_1, E_2, and E_3, on progesterone receptor levels and growth in the human breast cancer cell line MCF-7 in long-term culture. For progesterone receptor measurements, confluent cultures were exposed to estrogens for 48 h. Cytosol receptors were measured using protamine sulfate precipitation. All estrogens increased progesterone receptor levels. Estriol and E_4 achieved comparable stimulation to that observed with E_2, but about 10× and 50× higher concentrations, respectively, were required for E_3 and E_4. Growth stimulation was evaluated in cultures during the log phase of proliferation by tritiated thymidine incorporation. As for progesterone receptor stimulation, about 10× and 50× higher concentrations of E_3 and E_4, respectively, were needed to achieve equal stimulation of tritiated thymidine incorporation and to rescue cells from growth inhibition induced by antiestrogens. The authors concluded that both E_3 and E_4 behave as E_2 agonists but require substantially higher concentrations to achieve the effects of E_2.

3.3. Estrogen receptor binding studies

Tseng and Gurpide studied the competition of E_4 with E_2 for nuclear binding in human proliferative endometrium [45] Estetrol competed with E_2 for the same set of saturable binding sites with a relative binding affinity of 0.06, compared to a relative affinity of 0.33 for ethinyl estradiol (EE). However, in contrast to competition with EE, only about 65% of bound E_2 was exchanged by E_4 competition. The presence of specific saturable E_2 binding sites refractory to E_4 competition was interpreted as evidence for heterogeneity in the nuclear receptor population. A subsequent study [46] examined whether this heterogeneity was also found in cytosol, obtained from proliferative endometrium. As with nuclear binding, the relative affinity of E_4 for the cytosol receptor was low, showing values of 1.0, 0.7 and 0.015 for E_2, EE and E_4, respectively. However, in contrast to nuclear binding, heterogeneity was not detected in cytosolic E_2 receptors. Competitive receptor binding studies in rat uterine cytosol also revealed low estrogen receptor binding affinity for E_4, relative to that of E_2 [47].

4. Conclusions

Estetrol (E_4) was first discovered by Diczfalusy and co-workers in 1965. Studies by different investigators thereafter showed that this estrogenic steroid molecule with four hydroxyl groups is synthesized exclusively by the fetal liver during human pregnancy and reaches the maternal circulation through the placenta. Estetrol is minimally, if at all, metabolized and is not reconverted to estriol (E_3) or estradiol (E_2). When injected intravenously to adults, it was rapidly and completely excreted in urine as a Ring D monoglucuronide, but otherwise metabolically unaltered. According to this data, E_4 does not appear to enter the enterohepatic circulation and thus may be characterized by a simple, clearly definable metabolic pathway without intermediates.

Estetrol was detected in maternal urine as early as 9 weeks of pregnancy. It was found at high levels in maternal plasma during the second trimester of pregnancy, with steadily rising concentrations of unconjugated E_4 to about 1 ng/mL (>3 nmol/L) toward the end of pregnancy. Conjugated E_4 levels were seven times higher than unconjugated levels. The levels of unconjugated E_4 in fetal plasma at parturition were about 12–19 times those of maternal plasma. Amniotic fluid levels were about one-third of fetal plasma levels and 5–6 times higher than maternal plasma levels. Maternal urinary excretion in late pregnancy varied between 0.5 and 2.3 mg/day.

Estetrol produced a number of biological changes in the rodent uterus, such as weight increase, progesterone receptor stimulation, enzyme induction, and histological and ultrastructural changes. It also bound to the human endometrial estrogen receptor. The biochemical, histological and ultrastructural responses of the immature rat uterus to E_4 revealed a tendency toward cell differentiation, in contrast to the typical mitotic responses that were observed after E_2 administration.

After 20 years of experimental work, E4 research was virtually abandoned. In recent years based on new data, E_4 has experienced a *vita nova*, a revival of preclinical and clinical research activities with the goal to explore its potential for therapeutic use in humans. This review is intended to offer an historical account of the discovery of E_4 and the preclinical studies conducted during the heyday of E_4 research that ended in the mid-1980s.

From a teleological viewpoint it seems likely that an estrogenic steroid produced in such significant quantities by the male and female human fetal liver during pregnancy only is safe and has physiological significance. Ongoing and future research may elucidate this physiological role of E_4 during pregnancy to answer the question of the "raison d'être" of this intriguing steroid.

Recent new pharmacological and clinical data obtained since 2001 support the potential clinical use of E_4 for applications such as Hormone Replacement Therapy (HRT), contraception and prevention of osteoporosis [2,58,59], but more clinical research is required to confirm these and other possible treatment indications.

Conflict of interest

C.F.H. has financial interest in estetrol; H.C.B. is CEO and shareholder of Pantarhei Bioscience (PRB), the company developing estetrol.

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